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(54) Title: DIRECT CLONING OF PCR AMPLIFIED NUCLEIC ACIDS (57) Abstract Methods are described for producing recombinant DNA molecules from suitable host vectors and nucleic acids subjected to 3'-terminal transferase activity. In one embodiment, the method takes advantage of the single 3'-deoxy-adenosine monophosphate (dAMP) residues attached to the 3' termini of PCR generated nucleic acids. Vectors are prepared with recognition sequences that afford single 3'-terminal deoxy-thymidine monophosphate (dTMP) residues upon reaction with a suitable restriction enzyme. Thus, PCR generated copies of genes can be directly cloned into the vectors without need for preparing primers having suitable restriction sites therein. The invention also contemplates associated plasmid vectors and kits for implementing the methods.		

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DIRECT CLONING OF PCR AMPLIFIED NUCLEIC ACIDS

Description5 Technical Field

The present invention relates to the cloning of nucleic acids. More particularly, the invention relates to a simplified method for directly cloning polymerase generated nucleic acids into vectors.

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Background

In the short time that practical PCR techniques for amplifying nucleic acid material have been available, many applications have been advanced. For example, PCR has found numerous research applications, such as in the determination of genetic mutations, in engineering template-modified sequences using mismatched primer sequences, and in producing sufficient genetic material for direct sequencing. PCR has also been applied to many medical and legal problems where it has been used in such areas as diagnosis of monogenic diseases, analysis of biological evidence, etc. Further applications of PCR amplification are discussed in a number of references. See, e.g., PCR Technology, H. A. Erlich, ed., Stockman Press, 1989. Doubtless, many more applications will be forthcoming.

In PCR, specific nucleic acid target sequences are transcribed by a chain reaction in which a primer molecule complementary to a particular section of a nucleic acid template is used to form an extension product of the primer. Each primer extension product specifically anneals with a complementary primer molecule and the resulting primed template acts as a substrate for a further extension

reaction. These steps are repeated many times, preferably using an automated cycling procedure, thereby exponentially amplifying the initial nucleic acid material. PCR is particularly useful in detecting nucleic acid sequences that are initially present in only very small amounts. Procedures for conducting PCR have been extensively described. See, for example, U.S. Patent Nos. 4,683,195 and 4,683,202, both to Mullis, et al., which descriptions are incorporated herein by reference.

Modernly, PCR is performed by automated cycling of denaturation, oligonucleotide primer annealing to a genetic template, and primer extension using a thermostable DNA polymerase, e.g., the Tag enzyme isolated from the bacterium Thermus Aquaticus. See, e.g., U.S. Patent 4,889,818 issued to Gelfand et al, which description is incorporated herein by reference.

While PCR alone may be satisfactory for certain applications, e.g., direct sequencing, often it is desirable to obtain a clone of PCR amplified products for further analysis, modification, or synthesis of probes. For example, a number of mRNA species exhibit polymorphic transcripts. Alternative splicing of the mRNA species to give multiple transcripts can be unambiguously sequenced after molecular cloning of the PCR amplification products (Frohman et al., Proc. Natl. Acad. Sci. USA 85: 8998-9002 (1988)). Cloning of PCR generated samples to construct cDNA libraries may also be desired. Generally, a protocol entailing cloning of PCR products can be expected to generate a smaller set of products than PCR alone, thereby reducing the background associated with direct sequencing of PCR products.

The most common method for cloning PCR products involves incorporation of flanking restriction sites onto the ends of primer molecules. The PCR cycling is carried out and the amplified DNA is then purified, restricted with an appropriate endonuclease(s) and ligated to a compatible vector preparation. Thus, typical PCR cloning methods require preparation of PCR primer molecules attached to "add on" base sequences having a preferred restriction recognition sequence. Also, these methods can result in unintended internal restriction of uncharacterized or polymorphic sequences. Such limitations of previous methods add to the cost and complexity of cloning PCR products routinely.

Recently, it was reported that Taq polymerase catalyzes the nontemplated addition of single deoxyadenosine monophosphate (dAMP) residues to the 3' termini of blunt-ended DNA duplexes (Clark, J.M., Nucl. Acids Res. 20:9677-86 (1989)). Thus, Taq polymerase (and other thermostable polymerases) naturally creates restriction-like termini on DNA fragments. However, since these overhanging residues are widely viewed as incompatible with most molecular cloning schemes, the residues are routinely removed with nucleases to create blunt ends, such as S1, Klenow, and T4.

In view of the above considerations, a method is desired for directly cloning PCR products containing terminal 3'-dAMP residues into appropriate plasmids. Such method would eliminate the need for preparing primers having restriction recognition sequences and it would eliminate the need for a restriction step to prepare the PCR product for cloning. Additionally, such method would preferably allow cloning PCR products directly without an

intervening purification step.

Brief Summary of the Invention

5 The present invention is for a method of producing recombinant DNA nucleic acid molecules that include a DNA segment having terminal 3'-dAMP residues. Such DNA segments are generated by thermophilic polymerases during PCR amplification. Thus, in one embodiment of the invention, the method involves subjecting a target DNA segment to PCR amplification so that a plurality of double-stranded nucleic acids including the segment are formed with each double stranded nucleic acid having a single overhanging 3'- deoxyadenosine monophosphate (dAMP) residue. The double-stranded nucleic acids are admixed with a heterologous source of linear double-stranded DNA molecules, such as linearized plasmids, which are provided with a single overhanging deoxythymidylate (dTMP) residue at the 3' termini of the DNA molecules. The reaction mixture is maintained under predetermined ligation conditions to effect ligation of the 3'-dAMP-containing nucleic acids with the 3'-dTMP-containing DNA molecules to give the recombinant molecules.

25 In a preferred embodiment of the invention the target DNA segment will be a gene. In further preferred embodiments of the invention a suitable host cell line will be transformed with the gene and the gene will be maintained under predetermined reaction conditions which allow expression of the gene.

30 The present invention affords plasmids that comprise a first nucleotide sequence recognized by a restriction enzyme and a second nucleotide sequence recognized by a second restriction enzyme. Each recognition sequence is capable of generating a single

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terminal 3'-dTMP group upon reaction with the restriction enzymes. In a further preferred embodiment of the invention the restriction enzymes will be identical and either Xcm 1 or Hph 1. In further preferred embodiment, the DNA fragment that is removable upon restriction is synthesized as an oligonucleotide with additional restriction sites flanking the first set of restriction sites so that the oligonucleotide may be inserted readily into a suitable substrate.

Kits suitable for directly cloning PCR amplification products having 3'-dAMP residues into the instant plasmids are afforded by the present invention. The kits will include plasmids having at least two nucleotide sequences recognized by a restriction enzyme capable of generating single 3'-dTMP termini at each sequence. The kits will also include a restriction enzyme capable of cleaving the plasmids at each nucleotide sequence. Also, the kits may include DNA ligase capable of ligating the heterologous strands of PCR amplified DNA with the plasmids at the restriction sites.

The present invention thus affords novel methods and associated plasmids and kits for directly cloning recombinant DNA molecules from polynucleotides that have terminal 3'-deoxyAMP residues, such as those produced by PCR amplification. Amplified nucleic acids from genomic DNA, cDNA, or recombinant vectors, having DNA inserts, such as lambda phages, cosmids and YACS may be used. The instant cloning methods allow the generation of large amounts of genetic material via cell transformation, thereby making the direct sequencing of long nucleotide sequences feasible. Sequence information obtained by the present invention will be more reliable than previously available from

direct sequencing of PCR amplification products due to more specific ligation reactions and reduced numbers of restriction fragments. Such methods will be particularly useful in cDNA cloning efforts. The instant methods will also afford improved templates for synthesizing probes useful in genetic mapping and cloning projects, as well as hard copies of critical diagnostic samples, e.g., as in genetic fingerprinting. Also, the present invention affords reduced cost and simplicity to current PCR protocols due to elimination of the need for synthesizing primer sequences with specific recognition sequences, as well as the need for associated restriction steps.

Brief Description of the Drawings

Figure 1A depicts an exemplary cloning vector constructed according to the principles of the present invention. The genetic maps of host vector (pT7T319U) and cloned vector (pTA12) are shown.

Figure 1B presents a representative DNA sequence of plasmids constructed according to the principles of the present invention. The plasmids are provided with two Xcm 1 restriction sites between Hind III and Eco RI sites. Also the result of Xcm 1 digestion is shown as well as the result of ligation with a 3'-dAMP PCR amplified product.

Figure 2A presents a diagram showing the construction of a vector containing 2 Hph I sites. The vector is kanamycin resistant and has a color indicator system (β galactosidase).

Figure 2B presents a representative DNA sequence of plasmids constructed to include an Hph I restriction site on either side of an Eco RV recognition site. One Hph I site is present in the host vector while the second Hph I site is provided by

an inserted oligonucleotide. Also, the result of Hph I digestion is depicted as well as the result of ligation with terminal 3'-dAMP nucleic acids.

5 Detailed Description of the Invention

A. Definitions

Base Pair (bp): a partnership of adenine (A) with thymine (T), or of cytosine (C) with guanine (G) in a double stranded DNA molecule. In RNA, uracil (U) is substituted for thymine. Base pairs are said to be "complementary" when their component bases pair up normally when a DNA or RNA molecule adopts a double stranded configuration.

10 Complementary Nucleotide Sequence: a sequence of nucleotides in a single-stranded molecule of DNA or RNA that is sufficiently complementary to another single strand to specifically (non-randomly) hybridize to it with consequent hydrogen bonding.

15 Conserved: a nucleotide sequence is conserved with respect to a preselected (reference) sequence if it non-randomly hybridizes to an exact complement of the preselected sequence.

20 Duplex DNA: a double-stranded nucleic acid molecule comprising two strands of substantially complementary polynucleotides held together by one or more hydrogen bonds between each of the complementary bases present in a base pair of the duplex. Because the nucleotides that form a base pair can be either a ribonucleotide base or a deoxyribonucleotide base, the phrase "duplex DNA" refers to either a DNA-DNA duplex comprising two DNA strands (ds DNA), or an RNA-DNA duplex comprising one DNA and one RNA strand.

25 Gene: a nucleic acid whose nucleotide sequence codes for a RNA, DNA or polypeptide molecule. Genes may uninterrupted sequences of nucleotides or

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they may include such intervening segments as introns, promoter regions, splicing sites and repetitive sequences. A gene can be either RNA or DNA.

5 Hybridization: the pairing of complementary nucleotide sequences (strands of nucleic acid) to form a duplex, heteroduplex, or complex containing more than two single-stranded nucleic acids, by establishing hydrogen bonds between/among complementary base pairs. Hybridization is a specific, 10 i.e. non-random, interaction between/among complementary polynucleotides that can be competitively inhibited.

15 Hybridization product: the product (also called a "hybrid" or "duplex") formed when a polynucleotide hybridizes to a single or double-stranded nucleic acid. When a polynucleotide hybridizes to a double-stranded nucleic acid, the hybridization product formed is referred to as a triple helix or triple-stranded nucleic acid molecule. 20 Moser et al, Science, 238:645-50 (1987).

25 Nucleotide: a monomeric unit of DNA or RNA consisting of a sugar moiety (pentose), a phosphate group, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is a nucleoside. When the nucleoside contains a phosphate group bonded to the 3' or 5' position of the pentose it is referred to as a nucleotide. A sequence of operatively linked 30 nucleotides is typically referred to herein as a "base sequence" or "nucleotide sequence", and their grammatical equivalents, and is represented herein by a formula whose left to right orientation is in the conventional direction of 5'-terminus to 3'-terminus.

35 Nucleotide Analog: a purine or pyrimidine

nucleotide that differs structurally from an A, T, G, C, or U base, but is sufficiently similar to substitute for the normal nucleotide in a nucleic acid molecule. Inosine (I) is a nucleotide analog that can hydrogen bond with any of the other nucleotides, A, T, G, C, or U. In addition, methylated bases are known that can participate in nucleic acid hybridization.

Polynucleotide: a polymer of single or double stranded nucleotides. As used herein "polynucleotide" and its grammatical equivalents will include the full range of nucleic acids. A polynucleotide will typically refer to a nucleic acid molecule comprised of a linear strand of two or more deoxyribonucleotides and/or ribonucleotides. The exact size will depend on many factors, which in turn depends on the ultimate conditions of use, as is well known in the art. The polynucleotides of the present invention include primers, probes, RNA/DNA segments, oligonucleotides (relatively short polynucleotides), genes, vectors, plasmids, and the like.

Primer: a polynucleotide, whether purified from a nucleic acid restriction digest or produced synthetically, that is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product complementary to a template nucleic acid strand is induced, i.e., in the presence of nucleotides and an agent for polymerization, such as DNA polymerase, reverse transcriptase and the like, under suitable temperature and pH reaction conditions.

Recombinant DNA (rDNA) molecule: a DNA molecule produced by operatively linking a nucleic acid sequence, such as a gene, to a DNA molecule sequence of the present invention. Thus, a recombinant DNA molecule is a hybrid DNA molecule

comprising at least two nucleotide sequences not normally found together in Nature. rDNA's not having a common biological origin, i.e., evolutionarily different, are said to be "heterologous".

5 Vector: a DNA molecule capable of autonomous replication in a cell and to which a DNA segment, e.g., gene or polynucleotide, can be operatively linked so as to bring about replication of the attached segment. Vectors capable of directing the expression of genes encoding for one or more proteins are referred to herein as "expression vectors".
10 Particularly important vectors allow cloning of cDNA (complementary DNA) from mRNAs produced using reverse transcriptase.

15 B. DNA Segments

 In living organisms, the amino acid residue sequence of a protein or polypeptide is directly related via the genetic code to the deoxyribonucleic acid (DNA) sequence of the structural gene that codes for the protein. Thus, a structural gene can be defined in terms of the amino acid residue sequence, i.e., protein or polypeptide, for which it codes.

 An important and well known feature of the genetic code is its redundancy. That is, for most of the amino acids used to make proteins, more than one coding nucleotide triplet (codon) can code for or designate a particular amino acid residue. Therefore, a number of different nucleotide sequences can code for a particular amino acid residue sequence. Such nucleotide sequences are considered functionally equivalent since they can result in the production of the same amino acid residue sequence in all organisms. Occasionally, a methylated variant of a purine or
35 pyrimidine may be incorporated into a given nucleotide

sequence. However, such methylations do not affect the coding relationship in any way. A DNA target sequence of the present invention comprises no more than about 2000 nucleotide base pairs and may include
5 a structural gene. Usually, the DNA sequence is present as an uninterrupted linear series of codons where each codon codes for an amino acid residue, i.e., the DNA sequence contains no introns.

However, the invention also contemplates any
10 desired target fragment, such as a nucleic acid having an intervening sequence, a promoter, a regulatory sequence, a repetitive sequence, a flanking sequence, or a synthetic nucleic acid.

A DNA segment of the present invention can
15 easily be synthesized by chemical techniques, for example, via the phosphotriester method of Matteucci et al., J. Am. Chem. Soc., 103:3185 (1981). Of course, by chemically synthesizing the coding sequence, any desired modifications can be made simply
20 by substituting the appropriate bases for those encoding the native amino acid residue sequence.

The DNA segments of the present invention typically are duplex DNA molecules having cohesive termini, i.e., "overhanging" single-stranded portions
25 that extend beyond the double-stranded portion of the molecule. The presence of cohesive termini on the DNA molecules of the present invention is generally preferred.

Also contemplated by the present invention
30 are ribonucleic acid (RNA) equivalents of the above described DNA segments.

C. PCR Primers

The present invention contemplates using the
35 PCR technique described hereinbelow, to generate

enhanced amounts of a target nucleotide sequence. The PCR technique employs primer molecules to initiate the primer elongation reaction. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agents for polymerization. The exact lengths of the primers will depend on many factors, including temperature and the source of primer. For example, depending on the complexity of the template sequence, a polynucleotide primer typically contains 15 to 25 or more nucleotides, although it can contain fewer nucleotides. As few as 8 nucleotides in a polynucleotide primer have been reported as effective for use. Studier et al, Proc. Natl. Acad. Sci. USA, 86:6917-21 (1989). Short primer molecules generally require lower temperatures to form sufficiently stable hybridization complexes with template to initiate primer extension.

The primers used herein are selected to be "substantially" complementary to the different strands of each specific sequence to be synthesized or amplified. Accordingly, the primer must contain at its 3' terminus a nucleotide sequence sufficiently complementary to nonrandomly hybridize with its respective template strand. Therefore, the primer sequence may not reflect the exact sequence of the template. For example, a non-complementary polynucleotide can be attached to the 5' end of the primer, with the remainder of the primer sequence being substantially complementary to the strand. Such noncomplementary polynucleotides might code for a site for protein binding or simply be used to adjust the reading frame of the codons. Alternatively, noncomplementary bases or longer sequences can be interspersed into the primer, provided the primer

sequence has sufficient complementarity with the sequence of the template strand to allow non-random hybridization to occur so that an extension product can be formed under polynucleotide synthesizing conditions.

Sommer, et al., Nuc. Acid Res., 17:6749 (1989), reports that primers having as little as a 3 nucleotide exact match at the 3' end of the primer is capable of specifically initiating primer extension products, although less nonspecific hybridization occurs when the primer contains more nucleotides at the 3' end having exact complementarity with the template sequence. Therefore, a substantially complementary primer as used herein must contain at its 3' end at least 3 nucleotides having exact complementarity to the template sequence. A substantially complementary primer preferably contains at least 10 nucleotides, more preferably at least 18 nucleotides, and still more preferably at least 24 nucleotides, at its 3' end having the aforementioned complementarity. Still more preferred are primers whose entire nucleotide sequence have exact complementarity with the template sequence.

The choice of a primer's nucleotide sequence depends on factors such as the distance on the nucleic acid from the region coding for the desired specific nucleic acid sequence present in a nucleic acid of interest and its hybridization site on the nucleic acid relative to any second primer to be used.

The primer is preferably provided in single-stranded form for maximum efficiency, but may alternatively be double-stranded. If double-stranded, the primer is first treated to separate its strands before being used to prepare extension products.

Preferably, the primer is a polydeoxyribonucleotide.

Polynucleotides can be prepared by a variety of methods including de novo chemical synthesis and derivation of nucleic acid fragments from native nucleic acid sequences existing as genes, or parts of genes, in a genome, plasmid, or other vector, such as by restriction endonuclease digest of larger double-stranded nucleic acids and strand separation or by enzymatic synthesis using a nucleic acid template.

De novo chemical synthesis of a polynucleotide can be conducted using any suitable method, such as, for example, the phosphotriester or phosphodiester methods. See Narang et al, Meth. Enzymol., 68:90, (1979); U.S. Patent No. 4,356,270; Itakura et al, Ann. Rev. Biochem., 53:323-56 (1989); and Brown et al, Meth. Enzymol., 68:109, (1979).

Derivation of a polynucleotide from nucleic acids involves the cloning of a nucleic acid into an appropriate host by means of a cloning vector, replication of the vector and therefore multiplication of the amount of the cloned nucleic acid, and then the isolation of subfragments of the cloned nucleic acids. For a description of subcloning nucleic acid fragments, see Maniatis et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, pp 390-401 (1982); and see U.S. Patent Nos. 4,416,988 and No. 4,403,036.

D. Hybridization

Template nucleic acid sequences to be hybridized in the present methods can be present in any nucleic acid-containing sample so long as the sample is in a form, with respect to purity and concentration, compatible with nucleic acid hybridization reaction. Isolation of nucleic acids to a degree suitable for hybridization is generally known

and can be accomplished by a variety of means. For instance, nucleic acids can be isolated from a variety of nucleic acid-containing samples including body tissue, such as skin, muscle, hair, and the like, and body fluids such as blood, plasma, urine, amniotic fluids, cerebral spinal fluids, and the like. See, for example, Maniatis et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1982); and Ausubel et al, Current Protocols in Molecules Biology, John Wiley and Sons (1987).

The hybridization reaction mixture is maintained under hybridizing conditions for a time period sufficient for the primer to hybridize to complementary nucleic acid sequences present in the sample to form a hybridization product, i.e., a complex containing primer and template nucleic acid strands.

The phrase "hybridizing conditions" and its grammatical equivalents, when used with a maintenance time period, indicates subjecting the hybridization reaction admixture, in the context of the concentrations of reactants and accompanying reagents in the admixture, to time, temperature and pH conditions sufficient to allow the primer to anneal with the template sequence, typically to form a nucleic acid duplex. Such time, temperature and pH conditions required to accomplish hybridization depend, as is well known in the art, on the length of the primer to be hybridized, the degree of complementarity between the primer and the template, the guanosine and cytidine content of the polynucleotide, the stringency of hybridization desired, and the presence of salts or additional reagents in the hybridization reaction admixture as may affect the kinetics of hybridization. Methods for

optimizing hybridization conditions for a given hybridization reaction admixture are well known in the art.

Typical hybridizing conditions include the use of solutions buffered to pH values between 4 and 9, and are carried out at temperatures from 18C to 75C, preferably about 37C to about 65C, more preferably about 54C, and for time periods from 0.5 seconds to 24 hours, preferably 2 min.

Hybridization can be carried out in a homogeneous or heterogeneous format as is well known. The homogeneous hybridization reaction occurs entirely in solution, in which both the primer and template sequences to be hybridized are present in soluble forms in solution. A heterogeneous reaction involves the use of a matrix that is insoluble in the reaction medium to which either the primer or template is bound.

Also preferred are the homogeneous hybridization reactions such as are conducted for a reverse transcription of isolated mRNA or viral RNA to form cDNA, dideoxy sequencing and other procedures using primer extension reactions in which primer hybridization is a first step. Particularly preferred is the homogeneous hybridization reaction in which the template is amplified via a polymerase chain reaction (PCR).

Where the nucleic acid containing a template sequence is in a double-stranded (ds) form, it is preferred to first denature the dsDNA, as by heating or alkali treatment, prior to conducting the hybridization reaction. The denaturation of the dsDNA can be carried out prior to admixture with a primer to be hybridized, or it can be carried out after the admixture of the dsDNA with the primer. Where the

primer itself is provided as a double-stranded molecule, it too can be denatured prior to admixture, or it can be denatured concurrently with the template-containing dsDNA.

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E. Primer Extension Reactions

The primed template can be used to produce a strand of nucleic acid having a nucleotide sequence complementary to the template, i.e., a template-complement.

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If the template whose complement is to be produced is in the form of double stranded nucleic acid, it is typically first denatured, usually by melting, into single strands, such as ssDNA. The nucleic acid is then subjected to a (first) primer extension reaction by treating (contacting) the nucleic acid with a (first) polynucleotide synthesis primer having as a portion of its nucleotide sequence a sequence selected to be substantially complementary to a portion of the sequence of the template. The primer is capable of initiating a primer extension reaction by hybridizing to a specific nucleotide sequence, preferably at least about 8 nucleotides in length and more preferably at least about 20 nucleotides in length. This is accomplished by mixing an effective amount of the primer with the template nucleic acid, and an effective amount of nucleotide reactants to form a primer extension reaction admixture. The admixture is maintained under polynucleotide synthesizing conditions for a time period, which is typically predetermined, sufficient for the formation of a primer extension reaction product.

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The primer extension reaction is performed using any suitable method. Generally polynucleotide

synthesizing conditions are those wherein the reaction occurs in a buffered aqueous solution, preferably at a pH of 7-9, most preferably about 8. Preferably, a molar excess (for genomic nucleic acid, usually about 10⁶:1 primer:template) of the primer is admixed to the buffer containing the template strand. A large molar excess is preferred to improve the efficiency of the process. For polynucleotide primers of about 20 to 25 nucleotides in length, a typical ratio is in the range of 50 ng to 1 ug, preferably 250 ng, of primer per 100 ng to 500 ng of mammalian genomic DNA or per 10 to 50 ng of plasmid DNA.

The deoxyribonucleotide triphosphates (dNTPs) dATP, dCTP, dGTP, and dTTP are also admixed to the primer extension reaction admixture in amounts adequate to support the synthesis of primer extension products, and depends on the size and number of products to be synthesized. The resulting solution is heated to about 90C - 100C for about 1 to 10 minutes, preferably from 1 to 4 minutes. After this heating period the solution is allowed to cool to room temperature, which is preferable for primer hybridization. To the cooled mixture is added an appropriate agent for inducing or catalyzing the primer extension reaction, and the reaction is allowed to occur under conditions known in the art. The synthesis reaction may occur at from room temperature up to a temperature above which the inducing agent no longer functions efficiently. Thus, for example, if DNA polymerase is used as inducing agent, the temperature is generally no greater than about 40C unless the polymerase is heat-stable.

The inducing agent may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes.

Suitable enzymes for this purpose include, for example, E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, T7 DNA polymerase, recombinant modified T7 DNA polymerase, other available DNA polymerases, reverse transcriptase, and other enzymes, including heat-stable enzymes, which will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each nucleic acid strand.

Heat-stable (thermophilic) DNA polymerases are particularly preferred as they are stable in a most preferred embodiment in which PCR is conducted in a single solution in which the temperature is cycled. Representative heat-stable polymerases are the DNA polymerases isolated from Bacillus stearothermophilus (Bio-Rad, Richmond, CA), Thermus thermophilus (FINZYME, ATCC #27634), Thermus species (ATCC #31674), Thermus aquaticus strain TV 1151B (ATCC #25105), Sulfolobus acidocaldarius, described by Bukhrashuili et al, Biochem. Biophys. Acta, 1008: 102-7 (1989) and by Elie et al, Biochem. Biophys. Acta, 951:261-7 (1988), Thermus filiformis (ATCC #43280), the polymerase isolated from Thermus flavus (Molecular Biology Resources; Milwaukee, WI), and "Vent" polymerases (New England Biolabs, Beverly, MA). Particularly preferred is Taq DNA polymerase available from a variety of sources including Perkin Elmer Cetus, (Norwalk, CT), Promega (Madison, WI) and Stratagene (La Jolla, CA), and AmpliTaq™ DNA polymerase, a recombinant Taq DNA polymerase available from Perkin-Elmer Cetus.

In a preferred embodiment the polymerase selected will serve as a 3'-terminal transferase that is capable of placing a single, overhanging nucleotide

residue on a double-stranded nucleic acid. In a most preferred embodiment the transferase will attach a 3'-adenosine residue to an untemplated nucleic acid, such as performed by Taq polymerase. In alternative
5 embodiments, the transferase will add other nucleosides, e.g., guanosine, cytidine, thymidine.

This invention contemplates any nucleic acid segment produced by a polymerase either in vitro or in vivo. Any polymerase capable of producing a double-
10 stranded nucleic acid having a protruding 3' terminal AMP facilitating its insertion into a vector of the present invention is useful in practicing the invention. Particularly preferred are DNA dependent DNA polymerases and RNA dependent DNA polymerases.
15 DNA dependent DNA polymerases include the TAQ polymerase.

Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis
20 terminates, producing molecules of different lengths. There may be inducing agents, however, which initiate synthesis at the 5' end and proceed in the above direction, using the same process as described above.

The primer extension reaction product can
25 then be subjected to a second primer extension reaction by treating it with a second polynucleotide synthesis primer having a preselected nucleotide sequence. The second primer is capable of initiating the second reaction by hybridizing to a nucleotide
30 sequence, preferably at least about 8 nucleotides in length and more preferably at least about 20 nucleotides in length, found in the first product. This is accomplished by mixing the second primer, preferably a predetermined amount thereof, with the
35 first product, preferably a predetermined amount

thereof, to form a second primer extension reaction admixture. The admixture is maintained under polynucleotide synthesizing conditions for a time period, which is typically predetermined, sufficient for the formation of a second primer extension reaction product.

In preferred strategies, the first and second primer extension reactions are the first and second primer extension reactions in a polymerase chain reaction (PCR).

PCR is carried out by cycling, i.e., sequentially performing in one admixture, the above described first and second primer extension reactions, each cycle comprising polynucleotide synthesis followed by denaturation of the double stranded polynucleotides formed. Methods and systems for amplifying a specific nucleic acid sequence are described in U.S. Patents No. 4,683,195 and No. 4,683,202, both to Mullis et al; and the teachings in PCR Technology, Erlich, ed., Stockton Press (1989); Faloona et al, Methods in Enzymol., 155:335-50 (1987); and Polymerase Chain Reaction, Erlich et al, eds., Cold Spring Harbor Laboratories Press (1989).

F. PCR Cycling

PCR is carried out by cycling the following steps on one admixture: 1) denaturing step to form single-stranded templates, 2) hybridization step to hybridize primer to ss template, and 3) primer extension steps to form the extended product. PCR is performed in the above sequence (cycle) by changing the temperature of the PCR mixture to a temperature compatible with each step, in series.

The primer extension reaction conditions involve maintaining the reaction mixture for a time

period and at a temperature sufficient for a DNA polymerase primer extension reaction to occur to produce primer extension products as is well known. Conditions for conducting a primer extension reaction are well known. In a PCR format, the maintenance is carried out quickly to conveniently facilitate numerous cycles, in about 1 second to 5 minutes, preferably about 1.5 minutes, and at about 40C to 75C, preferably about 72C. Conducting at least one cycle of PCR results in the formation of amplified nucleic acid products. The PCR is typically conducted with at least 15 cycles, and preferably with about 20 to 40 cycles.

Hybridizing conditions were described earlier and are suitable for use in the PCR format. However, it is preferred and convenient to conduct hybridization in short periods of time, in 5 seconds to 12 minutes, preferably in 2 minutes, and in the temperature range of 30C to 75C, preferably about 40C to 65C, and more preferably about 54C.

G. Detection of PCR product

Detection of amplified nucleic acid product can be accomplished by any of a variety of well known techniques. In a preferred embodiment, the amplified product is separated on the basis of molecular weight by gel electrophoresis, and the separated products are then visualized by the use of nucleic acid specific stains which allow one to observe the discrete species of resolved amplified product present in the gel. Although numerous nucleic acid specific stains exist and would be suitable to visualize the electrophoretically separated nucleic acids, ethidium bromide is preferred.

Alternative methods suitable to detect the

amplified nucleic acid product include hybridization-based detection means that use a labeled polynucleotide probe capable of hybridizing to the amplified product. Exemplary of such detection means include the Southern blot analysis, ribonuclease protection analysis using in vitro labeled polyribonucleotide probes, and similar methods for detecting nucleic acids having specific nucleotide sequences. See, for example, Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, 1987.

In one approach for detecting the presence of a specific nucleic acid sequence, the deoxyribonucleotide triphosphates (dNTPs) used in the primer extension reaction include a label or indicating group that will render a primer extension product detectable. Typically such labels include radioactive atoms, chemically modified nucleotide bases, and the like.

Radioactive elements operatively linked to or present as part of a dNTP provide a useful means to facilitate the detection of a DNA primer extension product. A typical radioactive element is one that produces beta ray emissions. Elements that emit beta rays, such as ^3H , ^{14}C , ^{32}P , and ^{35}S represent a class of beta ray emission-producing radioactive element labels.

Alternatives to radioactively labeled dNTPs are dNTPs that are chemically modified to contain metal complexing agents, biotin-containing groups, fluorescent compounds, and the like.

One useful metal complexing agent is a lanthanide chelate compound formed by a lanthanide metal and beta-diketonate ligands, the lanthanide being bound to the nucleic acid or polynucleotide via

a chelate forming compound such as an EDTA-analogue so that a fluorescent lanthanide complex is formed. See U.S. Patents No. 4,374,120, and No. 4,569,790 and published International Patent Applications No. EP0139675 and No. WO87/02708.

Biotin or acridine ester-labeled oligonucleotides and their use in polynucleotides have been described. See U.S. Patent No. 4,707,404, published International Patent Application EP0212951 and European Patent No. 0087636. Useful fluorescent marker compounds include fluorescein, rhodamine, Texas Red, NBD and the like.

A labeled nucleotide residue present in a nucleic acid renders the nucleic acid itself labeled and therefore distinguishable over other nucleic acids present in a sample to be assayed. Detecting the presence of the label in the nucleic acid and thereby the presence of the specific nucleic sequence, typically involves separating the nucleic acid from any labeled dNTP that is not present as part of a primer extension reaction product.

Techniques for the separation of single stranded polynucleotide, such as non-hybridized labeled polynucleotide probe, from DNA duplex are well known, and typically involve the separation of single stranded from double stranded nucleic acids on the basis of their chemical properties. More often separation techniques involve the use of a heterogeneous hybridization format in which the non-hybridized probe is separated, typically by washing, from the DNA duplex that is bound to an insoluble matrix. Exemplary is the Southern blot technique, in which the matrix is a nitrocellulose sheet and the label is ³²P. Southern, *J. Mol. Biol.*, 98:503 (1975).

In another approach for detecting the

presence of a DNA duplex, and the one used herein as exemplary of a preferred embodiment, the DNA duplex is amplified as described herein and the resulting amplified nucleic acid product is detected.

5 Numerous applications of the PCR-based amplification method are contemplated that will be readily apparent to one skilled in the art. For example, cloning mRNA through reverse transcription to produce cDNA can be made more sensitive by the use of
10 PCR-based amplification of the produced cDNA. Insofar as nucleic acid sequencing can be conducted on PCR-amplified nucleic acid, the present invention can be used to improve sequencing of amplified nucleic acids.

15 H. Recombinant DNA Molecules

As is well known in the art, the choice of vector to which a DNA target sequence of the present invention is operatively linked depends upon the functional properties desired, e.g., protein
20 expression, and upon the host cell to be transformed. These limitations are inherent in the art of constructing recombinant DNA molecules. However, a vector contemplated by the present invention is at least capable of directing the replication, and
25 preferably also expression, of a gene operatively linked to the vector.

In preferred embodiments, a vector contemplated by the present invention includes a procaryotic replicon, i.e., a DNA sequence having the
30 ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a procaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition,
35 those embodiments that include a procaryotic replicon

may also include a gene whose expression confers drug resistance to a bacterial host transformed therewith. Typical bacterial drug resistance genes are those that confer resistance to ampicillin, tetracycline, or
5 kanamycin.

Those vectors that include a procaryotic replicon can also include a procaryotic promoter capable of directing the expression (transcription and translation) of the gene transformed therewith. A
10 promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction
15 sites for insertion of a PCR product molecule of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Bio-Rad Laboratories, (Richmond, CA) and pPL, pKK223, pTZ18U, pTZ19U, and pT7T319U, available from
20 Pharmacia, (Piscataway, NJ).

Expression vectors compatible with eucaryotic cells, preferably those compatible with vertebrate cells, can also be used to form the recombinant DNA molecules of the present invention.
25 Eucaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired gene. Typical of such vectors are pSVL
30 and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), and pTDT1 (ATCC, #31255).

In preferred embodiments, the eucaryotic cell expression vectors used to construct the recombinant DNA molecules of the present invention
35 include a selection marker that is effective in a

eucaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the gene whose expression results in neomycin resistance, i.e., the neomycin phosphotransferase (neo) gene. Southern et al., J. Mol. Appl. Genet., 1:327-341 (1982).

The use of retroviral expression vectors to form the rDNAs of the present invention is also contemplated. As used herein, the term "retroviral expression vector" refers to a DNA molecule that includes a promoter sequence derived from the long terminal repeat (LTR) region of a retrovirus genome.

In preferred embodiments, the expression vector is typically a retroviral expression vector that is preferably replication-incompetent in eucaryotic cells. The construction and use of retroviral vectors has been described by Sorge et al., Mol. Cell. Biol., 4:1730-37 (1984).

In a preferred embodiment of the present invention, the selected vector in its linearized form will have cohesive termini complementary to the termini generated by the polymerase, e.g., Taq polymerase, used in PCR amplification. Thus, a suitable vector preparation for directly cloning 3'-dAMP-terminal PCR products will be a plasmid having single, overhanging 3'-dTMP termini in its linearized form. (The dTMP moiety may have its monophosphate group attached to the 3' or, more preferably, to the 5' end of the thymidine nucleoside.)

Since thymine is not complementary with itself, the 3'-dTMP terminal vectors used will be derived from larger plasmids which include a removable linking fragment that joins the two 3'-dTMP groups until it is desired to form the linearized vector. The linearized vector will be generated from the

larger plasmid by restriction at the desired dTMP sites using the restriction enzymes described herein according to procedures well-known in the art and available from the appropriate enzyme supplier.

5 The present invention may be used to insert a cDNA fragment into a vector of the present invention. Preferably, the cDNA is inserted into the vector in a directional manner. This can be accomplished by employing a primer polynucleotide
10 containing a cleavage site such as a restriction endonuclease site to allow the protruding 3' AMP to be cleaved off of one end of the cDNA. This restriction endonuclease site is used to insert this restriction endonuclease-cleaved end of the cDNA into the vector.
15 The other end of the cDNA is inserted into the vector using the protruding 3'AMP present on that end of the cDNA to facilitate insertion into a vector of the present invention having a protruding 5' TMP. This procedure allows the cDNA to be inserted into a vector
20 in a directional manner facilitating the expression of the DNA cloned.

 In preferred embodiments, the primer containing the cleavage site employed to insert a cDNA into a vector of the present invention is a polymerase
25 chain reaction primer. Preferably, the second primer employed in the polymerase chain reaction does not contain the cleavage site. A vector of the present invention may be an expression vector, a cloning vector, a shuttle vector, and the like.

30 A most preferred embodiment of the invention will entail using a restriction enzyme capable of generating single, overhanging 3'-dTMP termini. However, only a few restriction enzymes are known that can be specified to generate such termini in plasmids,
35 i.e., Hph I, Mbo II, and Xcm I (New England Biolabs).

Moreover, two of these enzymes (Mbo II and Xcm I) show low ligation efficiencies (<20%). Also, two enzymes (Hph I and Mbo II) apparently find recognition sequences in virtually every known plasmid, e.g., each restricts the pUC19 plasmid at 7 sites, thereby producing many undesired fragments. Therefore, it may be desirable to screen the above enzymes for their activities and ligation efficiencies in particular applications. These methods are well known to those skilled in the art and are illustrated by the examples described hereinbelow.

A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini. For instance, complementary homo or heteropolymeric tracts can be added to the ends of the dAMP-terminal linking fragments that are removable from the vector with a restriction enzyme when it is desired to ligate the 3'-dAMP terminal products generated by PCR. Thus, the vector is covalently joined to the removable fragment via linking sequences and by hydrogen bonding between the complementary tails of the vector and linking sequence and between the linking sequences and removable fragment.

In a most preferred embodiment, the plasmid selected as vector will have Eco RI and Hind III restriction sites that allow insertion of a DNA sequence having at its termini complementary residues that allow restriction with Eco RI and Hind III. The internal region of the fragment will have two or more restriction sites recognized by the enzymes Xcm I, Hph I, or Mbo II, or a combination thereof, so that 3'-terminal dTMP residues can be generated for subsequent joining to 3'-dAMP terminal DNA segments. Restriction of the appropriate recognition sequences produces a removable DNA fragment and linearized plasmid. The

smaller DNA fragment can be removed by gel separation techniques well-known to those in the art.

5 Synthetic linkers containing a variety of restriction sites are commercially available from a number of sources including International Biotechnologies, Inc., New Haven, CT. Instructions for their use can be obtained from the supplier. Polynucleotide sequences, including the removable fragments and/or the linking sequences are preferably prepared by direct synthesis techniques such as those described for making PCR primer molecules.

Also contemplated by the present invention are RNA equivalents of the above described recombinant DNA molecules.

15 The 3'-dAMP nucleic acids are combined with linear DNA molecules in an admixture thereof and a ligase will be added to effect ligation of the components. Any ligase available commercially is contemplated to perform the ligation reaction effectively using methods and conditions well known to those skilled in the art. A preferred ligase is T4 DNA ligase.

20 Volume exclusion agents may also be used to accelerate the ligation reaction. However, such agents may cause excessive intramolecular circularizations in some cases.

Notably, it is also contemplated that certain nucleic acids desired to be cloned will have only one terminal 3'-dAMP residue per duplex. This situation may arise from incomplete reaction of a PCR polymerase, e.g., Taq, with amplified product. However, the present invention anticipates such eventuality, and will allow in some cases direct cloning of monoligated (linear) recombinants as long as the nucleic acid desired to be cloned binds to at

30

35

least one terminal 3'-dTMP residue presented by the vector. Additionally, transformation of cells with monoligated recombinant DNA molecules will likely occur normally in these cases with ligation at the second 3'-dTMP terminus occurring intracellularly.

I. Transformation of Cells

The present invention also relates to introducing the recombinant DNA molecules of the present invention into host cells, via a procedure commonly known as transformation or transfection. The host cell can be either procaryotic or eucaryotic. Bacterial cells are preferred procaryotic host cells and typically are a strain of E. coli such as, for example, the DH1αF strain. Preferred eucaryotic host cells include yeast and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Preferred eucaryotic host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61 and NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658. One preferred means of effecting transformation is electroporation.

Transformation of appropriate host cells with a recombinant DNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of procaryotic host cells, see, for example, Cohen et al., Proc. Natl. Acad. Sci. USA, 69:2110 (1972); and Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). With regard to transformation of vertebrate cells with retroviral vectors containing rDNAs, see, for example, Sorge et al., Mol. Cell. Biol., 4:1730-37 (1984);

Graham et al., Virology, 52:456 (1973); and Wigler et al., Proc. Natl. Acad. Sci. USA, 76:1373-76 (1979).

J. Assays for Recombinant Vectors

5 Successfully transformed cells, i.e., cells
that contain a recombinant DNA molecule of the present
invention, can be identified by well known techniques.
For example, cells resulting from the introduction of
an rDNA of the present invention can be cloned to
10 produce monoclonal colonies. Cells from those
colonies can be harvested, lysed and their DNA content
examined for the presence of the rDNA using a method
such as that described by Southern, J. Mol. Biol.,
98:503 (1975) or Berent et al., Biotech., 3:208
15 (1985).

 In addition to directly assaying for the
presence of rDNA, successful transformation can be
confirmed by well known immunological methods when the
rDNA is capable of directing the expression of a
20 subject polypeptide. For example, cells successfully
transformed with a subject rDNA containing an
expression vector produce a polypeptide displaying a
characteristic antigenicity. Samples of a culture
containing cells suspected of being transformed are
25 harvested and assayed for a subject polypeptide using
antibodies specific for that polypeptide antigen, such
as those produced by an appropriate hybridoma.

 Thus, in addition to the transformed host
cells themselves, the present invention also
30 contemplates a culture of those cells, preferably a
monoclonal (clonally homogeneous) culture, or a
culture derived from a monoclonal culture, in a
nutrient medium. Nutrient media useful for culturing
transformed host cells are well known in the art and
35 can be obtained from several commercial sources. In

embodiments wherein the host cell is mammalian, a "serum-free" medium is preferably used.

The present method entails a culture comprising a nutrient medium containing host cells transformed with a recombinant DNA molecule of the present invention that is capable of expressing a gene encoding a subject polypeptide. The culture is maintained for a time period sufficient for the transformed cells to express the subject polypeptide. The expressed polypeptide is then recovered from the culture.

Methods for recovering an expressed polypeptide from a culture are well known in the art and include fractionation of the polypeptide-containing portion of the culture using well known biochemical techniques. For instance, the methods of gel filtration, gel chromatography, ultrafiltration, electrophoresis, ion exchange, affinity chromatography and the like, such as are known for protein fractionations, can be used to isolate the expressed proteins found in the culture. In addition, immunochemical methods, such as immunoaffinity, immunoabsorption and the like can be performed using well known methods.

A preferred embodiment of the present invention will employ the Lac Z' blue/white phenotype of E. coli to allow a visual assay for effective cloning. To use this assay method restriction sites are engineered in-phase with the Lac Z' reading frame. Thus, a DNA fragment inserted into this indicator gene disrupts normal translation of the β -galactosidase protein resulting in white colony phenotypes on growth media containing the chromogenic dye XGAL.

Preferred embodiments of the present invention contain antibiotic resistance genes such as

ampicillin resistance gene or kanamycin resistance genes. While these particular kanamycin resistance genes are preferred, other equivalent genes and plasmid such as the genes in the kanamycin resistance Gene Block sold by Pharmacia, or the kanamycin resistance plasmid pMON 530, and the like will be useful.

K. Compositions and Kits

Many of the compounds and groups involved in the instant specification (e.g., nucleic acids) have a number of forms, particularly variably protonated forms, in equilibrium with each other. As the skilled practitioner will understand, representation herein of one form of a compound or group is intended to include all forms thereof that are in equilibrium with each other.

In the present specification, "uM" means micromolar, "ul" means microliter, and "ug" means microgram.

The compositions serving as host vectors can be packaged in kit form. As used herein, the term "package" refers to a solid matrix or material customarily utilized in a system and capable of holding within fixed limits one or more of the reagent components for use in a method of the present invention. Such materials include glass and plastic (e.g., polyethylene, polypropylene and polycarbonate) bottles, vials, paper, plastic and plastic-foil laminated envelopes and the like. Thus, for example, a package can be a glass vial used to contain the appropriate quantities of polynucleotide primer(s), plasmids, restriction enzyme(s), DNA polymerase, DNA ligase, or a combination thereof. An aliquot of each component sufficient to perform at least one program

of cloning will be provided in each container.

In preferred embodiments, the vector is operatively linked to a label, e.g., indicator gene, thereby providing a means to detect a DNA segment incorporated into a target plasmid. Preferred labels are well known to those skilled in the art and include those discussed hereinbefore, especially the Lac Z' visual marker. Other selectable markers may be used and are well known in the art.

Kits useful for producing a template-complement or for amplification or detection of a specific nucleic acid sequence using a primer extension reaction methodology also typically include, in separate containers within the kit, dNTPs where N is adenine, thymine, guanine and cytosine, and other like agents for performing primer extension reactions.

The reagent species, indicating means or primer extension reaction reagents of any system described herein can be provided in solution, as a liquid dispersion or as a substantially dry powder, e.g., the plasmids may be provided in lyophilized form. Where the reagent species or indicating means is an enzyme, the enzyme's substrate can also be provided in a separate package of a system. A solid support and one or more buffers can also be included as separately packaged elements in this system.

The present invention is more fully understood by the specific examples described hereinbelow, and by the appended claims.

Examples

1. Preparation of Modified Plasmids

The starting plasmids selected for vector construction were of the pT7T319U variety (Mead et al., Protein Engineering, 1:67-74 (1986)), which is a

derivative of pTZ19U and is available from Pharmacia LKB.

Complementary single strands of nucleic acids were synthesized with an automatic DNA synthesizer, Pharmacia "Gene Assembler", using the manufacturer's recommended conditions. The single stranded sequences were allowed to hybridize thereby giving a complementary asymmetric DNA duplex having the nucleotide sequence shown in Figure 1. This oligonucleotide allows digestion by Xcm I at two sites (between residues 18 and 19 on the upper chain and between residues 14 and 15 on the lower chain) to form a single, overhanging dTMP terminus on the 3' end of each strand.

The pT7T319U plasmids and oligonucleotides prepared above were separately treated with Hind III and Eco RI restriction enzymes using reaction conditions recommended by the enzyme supplier, New England Biolabs. The restriction sites involving pT7T319U were selected so that the Lac Z' reading frame of the plasmids would be interrupted by any insert provided therein, thereby allowing a visual assay according to the well known blue/white phenotype of B-galactosidase.

The linearized plasmids were separated from from the small fragments released between the Eco RI and Hind III sites by electrophoresis on a 1% agarose gel and the linearized plasmids were removed from the gel by electroelution. Similarly, the central sequence of the oligonucleotide was separated from its flanking segments released by Eco RI and Hind III.

The purified linearized plasmids and oligonucleotides were admixed in the ratio of 1:1 to 1:3, and ligated with T4 DNA ligase (New England Biolabs, Beverly, MA) under reaction conditions

described by the supplier. The recombinant plasmids so formed are designated pTA12 herein. Figure 1A depicts the gene maps of the host and pTA12 plasmids.

2. Preparation of Cloning Vector

pTA12 plasmids prepared as in Example 1 were digested with Xcm 1 according to procedures recommended by the supplier, New England Biolabs. The small fragment between the two Xcm 1 restriction sites (see Figure 1B) was separated from the large host fragment by electrophoresis on a 1% agarose gel followed by electroelution, or, in some cases, by differential precipitation with isopropanol.

The isolated linearized pTA12, designated pTA12-L, was ligated separately with each of the following unpurified PCR amplification products: (1) ribosomal RNA genes amplified from genomic DNA of Sarcophaga bullata, (2) ribosomal RNA genes amplified from genomic DNA of Vairimorpha necatrix, and (3) myosin heavy chain gene amplified from genomic DNA of Caenorhabditis elegans.

The primers used in generating the PCR amplified nucleic acids were the following:

C. elegans

CCTGGGCGACGAACCAAGTAA
CGCCACCAAGGGAGACCAGG

S. Bullata

CTGGTTGATCCTGCCAG
GGTTACCTTGTTACGACTT

V. necatrix

GGAGGAAAAGAACTAAC
TTGGAGACCTGCTGCGG

The ligation reactions were performed using T4 DNA ligase under the following conditions:

25mM Tris-HCl (pH 7.8)

10mM MgCl₂

1mM DTT

1mM ATP

5

50-100ng vector DNA (gel purified Xcm I
digested pTA 12)

1-9μl PCR reaction products (unpurified)

1μl T4 DNA ligase (New England Biolabs)

10

Each reaction admixture was maintained at
16°C for 16-24 hours. The reaction products formed
were used to transform cells for assay of cloning
efficiency as described hereinbelow with the results
presented in Table 1.

15

Table 1

Vector End (plasmid+ enzyme)	Insert	Colony Blue	Phenotype White	white total	Colonies with inserts
T-extended (50ng PTA12/Xcm I)	none (self-ligated)	2660	30	1.1%	ND
T-extended (50ng PTA12/Xcm I)	PCR (10ng S. bullata)	368	65	15.0%	23/24
T-extended (50ng PTA12/Xcm I)	PCR (10ng V. necatrix)	256	27	9.5%	23/24
T-extended (50ng PTA12/Xcm I)	PCR (10ng C.elegans)	608	78	11.4%	8/10
T-extended (50ng PTA12/Xcm I)	blunt (125ng M13mp18/Alu I)	4100	60	1.4%	3/6
blunt (150ng PTZ18U/Sma I)	none (self-ligated)	3000	40	1.1%	ND
blunt (150ng PTZ18U/Sma I)	PCR (10ng S.bullata)	4140	120	2.8%	0/22
blunt (50ng PTZ18U/Sma I)	PCR (10ng C.elegans)	15520	3920	20.2%	7/96
blunt (150ng PTZ18U/Sma I)	blunt (125ng M13mp18/Alu I)	2120	4530	68%	ND

3. Transformation and Assay of Xcm 1 Generated Vectors

Approximately 10 μ L of each ligation admixture prepared in Example 2 was individually used to transform a competent DH1aF strain of E. coli. The transformed cells were then plated on media containing XGAL.

The number of blue and white colonies were recorded and selected white colonies were analyzed for inserted DNA fragments by purifying small amounts of the plasmid DNA and restricting with the appropriate restriction enzyme. The results are presented in Table 1. A total of 120 white colonies were assayed for inserts and 108 (90%) contained fragments corresponding to the original PCR products.

The ratio of white colonies to blue colonies obtained from the above experiments range from 0.01 to 0.15. The calculated cloning efficiencies were approximately $1-5 \times 10^4$ colonies/ μ g of PCR product. Optimal cloning efficiencies of approximately 12% white/total number of colonies using competent cells able to yield 10^8 cells/ μ g of supercoiled DNA were obtained.

The results presented in Table 1 afford several important observations. First, the ligation results for blunt-ended pTZ18U restricted with Sma I and blunt-ended M13mp18 restricted with Alu I indicate that the chosen ligation conditions are favorable for ligation. Second, a comparison of the cloning efficiencies of S. bullata and C. elegans PCR products with Xcm1 digested pTA12 (T-extended) plasmids with the efficiencies of cloning to Sma I digested pTZ18U (blunt) plasmids reveals a much higher cloning efficiency with the T-extended plasmids. Third, the X-cm 1 digested pTA12 plasmids even show good cloning

efficiencies with blunt-ended fragments, e.g.,
M13mp18/Alu 1.

4. Assay of Hph 1 Generated Vectors

5 Recombinant vectors including two Hph 1
restriction sites were prepared using similar
techniques to those for construction of the Xcm 1
vectors described above.

10 Briefly, PTA112 was derived from the PHSS6
(Figure 2A) described by in Proc. Natl. Acad. Sci.,
U.S.A., 83:735-739 (1986) in the following manner.
The pH 556 plasmid was digested with the restriction
endonuclease Not I. The resulting fragment containing
15 the kanamycin resistance gene and the origin of
replication was isolated.

A Hae III fragment containing the PUC 19
polylinker region and the β galactosidase gene was
isolated from PUC 19 by restriction digestion with Hae
III. Not I linkers (New England Biolabs) were ligated
20 to the Hae III fragment. After removing the extra Not
I linkers by restriction endonuclease digestion this β
galactosidase containing fragment was ligated inserted
to the kanamycin resistance gene containing fragment
isolated above to produce pH SS6* (Figure 2A).

25 pH SS6* was mutagenized using the oligo
primers and the standard polymerase chain reaction
mutagenesis procedure described in PCR Protocols,
Academic Press, New York (1990) to remove the Hph I
site present in the kanamycin resistance gene. This
30 mutagenesis procedure produced pTA (Figure 2A).

The polylinker of the PTA was replaced with
the Hph I-containing polylinker shown in Figure 2B by
digesting pTA with Eco RI and Eco RV restriction
endonucleases and ligating the Hph I-containing
35 polylinker to it to produce pTA 112. pTA 112 was

linearized with Hph I and the resulting large fragment purified and designated pTA 112-L.

The cDNA fragments were generated by PCR amplification of MS2 RNA, supplied by Boehringer Mannheim (Mannheim, West Germany). cDNA templates of the RNA were prepared following the procedure described by Gülder, U. et al., Gene, 25:263 (1983). The cDNA precursors were amplified via PCR using forward and reverse primers corresponding to the terminal sequences of the cDNA fragments. The primers were synthesized on a Pharmacia "Gene Assembler". The forward primer had the sequence 5'-CCTTAGGTTCTGGTAATGAC-3' and the reverse primer had the sequence 5'-GGGTGCAATCTCACTGGGAC- 3'.

PCR amplification conditions followed those recommended by the manufacturer and were:

100ng plasmid (900 bp MS2)
0.2 µg primer (forward/reverse)
1 µL 25mM dNTP's
5 µL 10 x PCR buffer
50 µL total volume

Cycles: (1) 94C, 1 min; 55C, 2 min; 72C, 3 min
(2) 72C, 7 min;
(3) 25C, 10 min.

The fragments were amplified by PCR using three different polymerases: (1) "Taq" polymerase, isolated from Thermus aquaticus and obtained from Perkin Elmer Cetus, (2) "Vent" polymerase, purchased from New England Biolabs, and (3) the thermophilic polymerase, "Thermo", isolated from Thermus flavus, and obtained from Molecular Biology Resources. These unpurified PCR products were ligated with an Hph 1 analog of pTA12, hereinbelow called pTA112, using procedures described above for Xcm 1 recognized plasmids. The

nucleotide sequence for the synthetically prepared oligonucleotide having Eco RI and Eco RV termini is shown in Figure 2, as is the result of Hph 1 digestion and ligation with 3'-dAMP PCR products.

5 The results presented in Table 2 allow comparisons of cloning efficiencies for Hph 1 digested (T-extended) pTA112 plasmids and blunt-ended control plasmids (pTA112 digested with Eco RV) using unpurified PCR amplified MS2 which was generated with
10 three different polymerases.

 Thus, Hph 1 digested plasmids show near 100% cloning efficiencies with each polymerase studied and over a 3-fold range in PCR product ratio. Most significantly, the reactions of T-extended (Hph 1
15 digested) plasmids and of blunt-ended (Eco RV digested) plasmids of Taq-generated MS2 products indicate that the d-AMP terminal MS2 products are more than ten times more efficiently cloned into the T-extended plasmids than the blunt-ended vectors.

20 5. Representative Kit

 A representative kit for use in directly cloning DNA segments having terminal 3'-dAMP residues includes one or more, and preferably all, of the
25 following components in separate containers:

Box 1: Description

Component

Designation

TA1	Sterile water: 1mL
30 TA2	10 x ligation buffer: 100 μ L
TA3	Ligation-ready cloning vector: 1.1 μ g (lyophilized pTA112-L or pTA12-L)
TA4	1 x Tris EDTA buffer: 100 μ L
TA5	T4 DNA ligase: 22 μ L
35 TA6	Control vector: 1 μ g/10 μ L Tris EDTA

TA7 Forward primer: (0.2 $\mu\text{g}/\mu\text{L}$) : 5 μL
TA8 Reverse primer: (0.2 $\mu\text{g}/\mu\text{L}$) : .5 μL
TA9 10 x PCR buffer: 100 μL
TA10 25 mM dNTPs (deoxynucleoside triphosphates)
5 TA11 0.5 M BME
TA12 Supercoiled plasmid pUC18 (control): 10 μL

Box 2:

TA13 21 aliquots of competent E. coli cells
(strain JM109 or NM522 are preferred) at 50
10 μL
TA14 SOC Medium for culturing said cells about 20
ml (p A.2 of Molecular Cloning: A
Laboratory Manual, Second Edition, Sanbrook,
et al., Cold Spring Harbor Press (1989))
15 The above kit will provide sufficient
reagents for performing 20 cloning reactions.

In one preferred embodiment, the kit
includes at least components TA3, TA6, TA7, and TA8.

In another preferred embodiment, the kit
20 includes at least components TA3, TA4, TA6, TA7, TA8,
TA13, and TA14.

In a further preferred embodiment, the kit
includes at least components TA3 and TA13.

25 Although the present invention has been
described in some detail by way of illustration and
example for purposes of clarity and understanding, it
will be obvious that certain modifications may be
practiced within the scope of the appended claims.
Therefore, the scope of the invention, and equivalents
30 thereof, should be determined with reference to the
appended claims rather than with reference to the
above description.

Table 2

Vector End (plasmid+ enzyme)	Insert	Colony Blue	Phenotype White	White total	Colonies with inserts(%)
T-extended (50ng pTA112/Hph I)	none	73	3	3.9%	N/D
T-extended (50ng pTA112/Hph I)	Taq-PCR (16ng MS2)	78	13	14.3%	>95
T-extended (50ng pTA112/Hph I)	Vent-PCR (16ng MS2)	85	17	16.7%	>95
T-extended (50ng pTA112/Hph I)	Thermo-PCR (16ng MS2)	113	18	13.7%	>95
T-extended (50ng pTA112/Hph I)	Taq-PCR (48ng MS2)	84	20	19.2%	>95
T-extended (50ng pTA112/Hph I)	Vent-PCR (48ng MS2)	89	20	19.5%	>95
T-extended (50ng pTA112/Hph I)	Thermo-PCR (48ng MS2)	71	18	18.2%	>95
blunt-ended (50ng pTA112/Eco RV)	none	1500	15	1.0%	N/D
blunt-ended (50ng pTA112/Eco RV)	Taq-PCR (16ng MS2)	2000	42	1.2%	N/D
blunt-ended (50ng pTA112/Eco RV)	Taq-PCR (48ng MS2)	2000	50	2.4%	N/D

WHAT IS CLAIMED IS:

1. A method for producing a plurality of recombinant DNA molecules each including a target DNA segment,
5 which method comprises:

(a) forming a plurality of double-stranded nucleic acids including said DNA segment, each of said nucleic acids having a single, overhanging 3'-dAMP residue at each terminus of the nucleic acid;

10 (b) forming a ligation reaction admixture by admixing said plurality of double-stranded nucleic acids with linear double-stranded DNA molecules, said DNA molecules each having a single, overhanging 3'-dTTP residue at each terminus of the DNA molecules;
15 and

(c) maintaining said admixture under ligation reaction conditions for a time period sufficient to effect ligation of said nucleic acids to said DNA molecules to produce said recombinant DNA
20 molecules.

2. A method as in claim 1, wherein the DNA segment includes a gene.

25 3. A method as in claim 1, wherein the double-stranded nucleic acids are produced by polymerase chain

reaction (PCR) amplification.

4. A method as in claim 2, further comprising transforming a suitable host cell line with the
5 recombinant DNA molecules.

5. A method as in claim 4, further comprising incubating the cells for a sufficient time to allow expression of the gene.

10

6. A method for producing recombinant DNA nucleic acid molecules each including a target DNA segment, which method comprises:

(a) subjecting a target nucleotide sequence
15 including said DNA segment to polymerase chain reaction (PCR) amplification to produce a plurality of double-stranded nucleic acids including said DNA segment, each of said nucleic acids having a single, overhanging (unpaired) dAMP residue located at a 3'
20 terminus of the nucleic acid;

(b) forming a ligation reaction admixture by admixing said plurality of double-stranded nucleic acids with linear double-stranded DNA molecules, said DNA molecules each having a single, overhanging
25 (unpaired) dTMP residue at each 3' terminus of the DNA molecules; and

(c) maintaining said admixture under predetermined reaction conditions for a time period sufficient to effect ligation of said nucleic acids to said DNA molecules to produce said recombinant DNA nucleic acid molecules.

7. A method as in claim 6, wherein the DNA segment includes a gene.

8. A method as in claim 6, further comprising transforming a host cell line with the recombinant DNA molecules.

9. A method as in claim 8, further comprising incubating the cells for a sufficient time to allow expression of the gene.

10. A method for producing a plurality of recombinant DNA molecules each including a target DNA segment, which method comprises:

(a) subjecting a target nucleotide sequence including said DNA segment to polymerase chain reaction (PCR) amplification using a thermophilic polymerase having 3'-terminal adenosine transferase activity to produce a plurality of double-stranded nucleic acids including said DNA segment, each of said

nucleic acids having a single, overhanging 3'-dAMP residue at each terminus of the nucleic acid;

(b) reacting a plurality of plasmids with a restriction enzyme that recognizes at least two nucleotide sequences within each of said plasmids so that a plurality of linear DNA molecules are formed, each of said molecules having a single, overhanging 3'-dTMP residue at each terminus;

(c) combining said double-stranded nucleic acids and said linear DNA molecules with a preselected ligase enzyme to form an admixture thereof;

(d) maintaining said admixture under predetermined reaction conditions for a time period sufficient to effect ligation of said nucleic acids to said DNA molecules to produce said recombinant DNA molecules.

11. A method as in claim 10, further comprising transforming suitable host cells with recombinant DNA molecules carrying a selectable marker.

12. A method as in claim 10, further comprising separating said linear DNA molecules in step (b) from smaller restriction fragments prior to performing step (c).

13. A plasmid comprising a first nucleotide sequence
recognized by a first restriction enzyme and a second
nucleotide sequence recognized by a second restriction
enzyme with each enzyme capable of generating a single
5 terminal 3'-dTMP group upon reaction with the first
and second nucleotide sequences.

14. A plasmid as in claim 13, wherein the first and
second nucleotide sequences each define a recognition
10 sequence for the restriction enzyme Xcm I or Hph I.

15. A plasmid as in claim 14, wherein the nucleotide
sequences are identical.

16. A kit for introducing a nucleotide sequence into a
plasmid, which kit comprises an enclosure containing,
in separate containers:

an aliquot of plasmids, said plasmids each
having at least two nucleotide sequences recognized by
20 a restriction enzyme capable of generating a single,
overhanging 3'-dTMP group; and

an aliquot of said restriction enzyme
capable of recognizing each of said plasmids at said
nucleotide sequence and cleaving said plasmids, said
25 restriction enzyme being present in the aliquot in an
amount sufficient to cleave at least a portion of said

plasmids.

17. A kit as in claim 18, wherein the enzyme is Xcm I or Hph I.

5

18. A kit as in claim 18, wherein the plasmids are lyophilized.

19. A kit as in claim 18, wherein said enclosure
10 further contains in a separate container an aliquot of a ligase capable of ligating a nucleic acid sequence into each of said plasmids at said overhanging 3'-dTTP groups.

15 20. A kit as in claim 18, wherein said enclosure further contains in a separate container an aliquot of primer molecules for use in PCR amplification of a target DNA sequence.

20 21. A kit as in claim 18, wherein said enclosure further contains in a separate container an aliquot of control plasmids.

22. A kit as in claim 18, wherein said enclosure
25 further contains in a separate container an aliquot of a 3'-terminal transferase.

23. A kit for introducing a nucleotide sequence into a plasmid, which kit comprises an enclosure containing, in separate containers:

- 5 an aliquot of lyophilized cloning plasmids, said plasmids each having at least two nucleotide sequences recognized by a restriction enzyme capable of generating a single, overhanging 3'-dTMP; and
- 10 an aliquot of competent host cells capable of replicating said plasmids upon transformation therewith.

24. A kit for introducing a nucleotide sequence into a plasmid, which kit comprises an enclosure containing, in separate containers:

- 15 an aliquot of linear plasmid DNA having single, overhanging 3'-dTMP termini;
- 20 an aliquot of a control vector containing a DNA insert, said control vector being present in an amount permitting polymerase chain reaction (PCR) amplification of said DNA insert using a thermostable enzyme having 3'-terminal adenosine transferase activity;
- 25 a separate aliquot of each of forward and reverse PCR primers suitable for PCR amplifying said DNA insert from said cloning vector; and

an aliquot of a DNA ligase, said DNA ligase being present in an amount sufficient to ligate a DNA insert produced by PCR amplifying the DNA insert of said control vector using said forward and reverse PCR primers and a thermostable polymerase having 3'-terminal adenosine transferase activity.

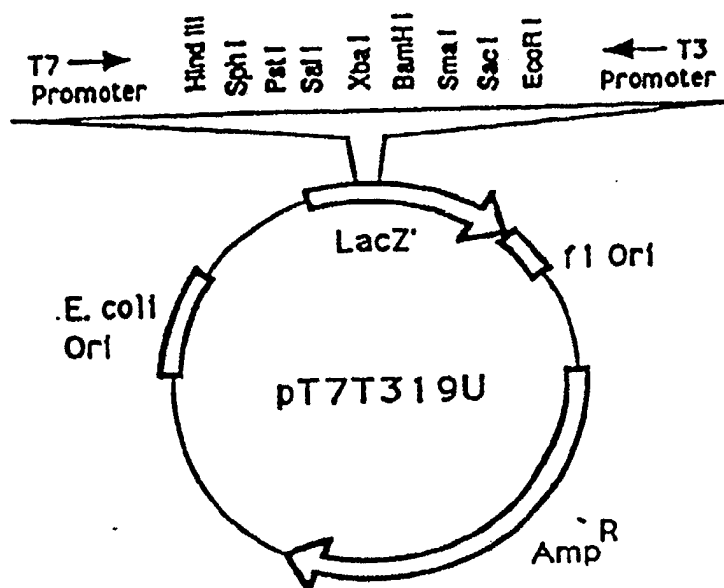
25. The kit of claim 24 wherein said linear plasmid DNA is pTA112-L.

26. The kit of claim 25 wherein said pTA112-L is in lyophilized form.

27. The kit of claim 24 wherein said linear plasmid DNA is pTA12-L.

28. The kit of claim 27 wherein said linear plasmid DNA is in lyophilized form.

FIGURE 1A



↓ Restrict with EcoR I + Hind III;
remove central fragment

↓ Ligate Xcm I oligonucleotides

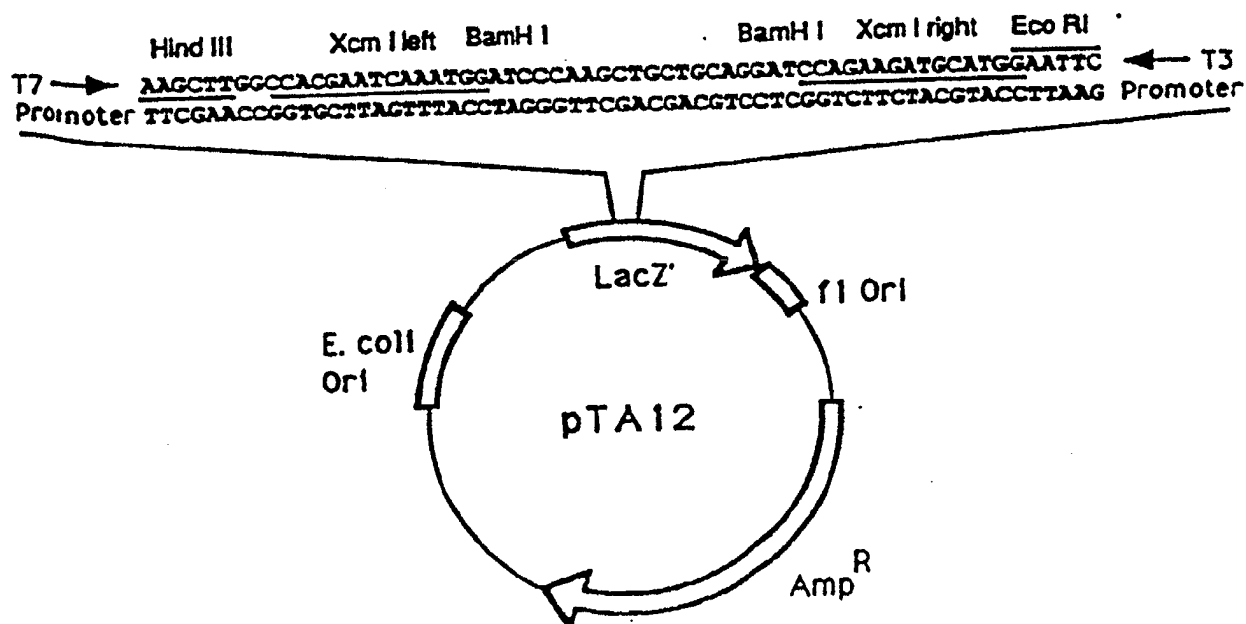
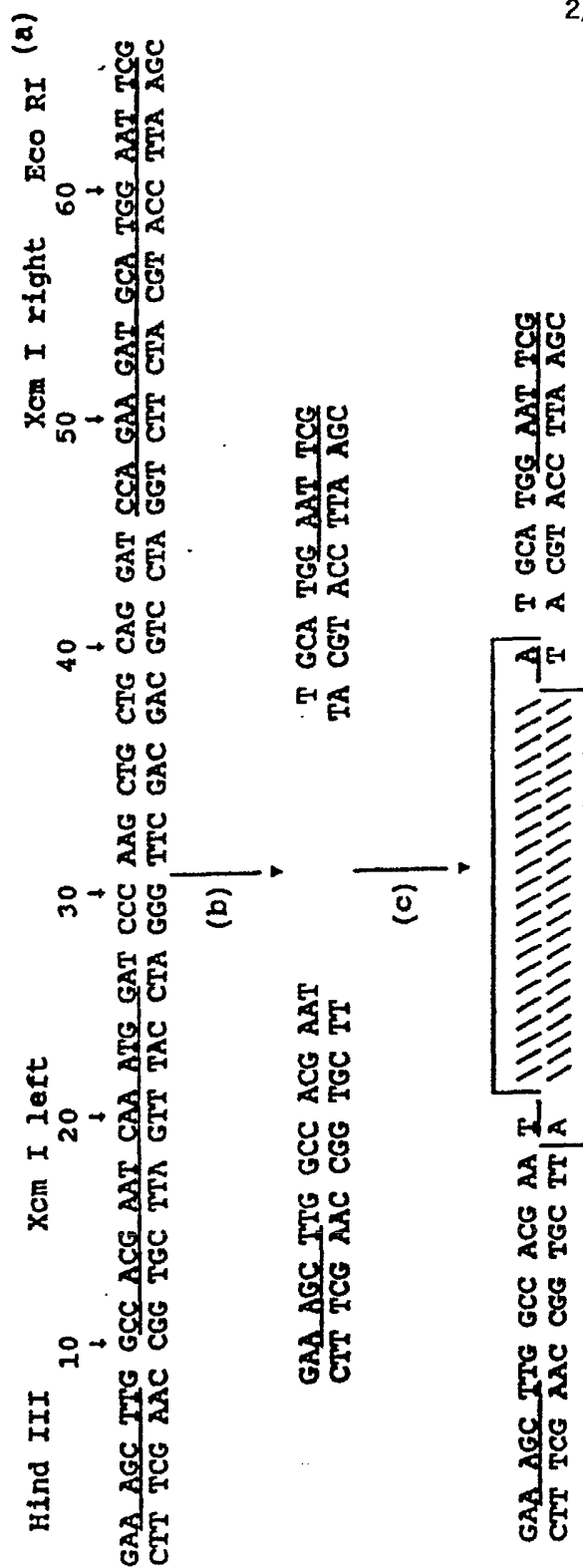


FIGURE 1B



(a) underlined regions indicate restriction recognition sequences

(b) Xcm I digest, remove central fragment

(c) PCR amplified product (3'-dAMP) admixed; T4 DNA ligase.

FIGURE 2A

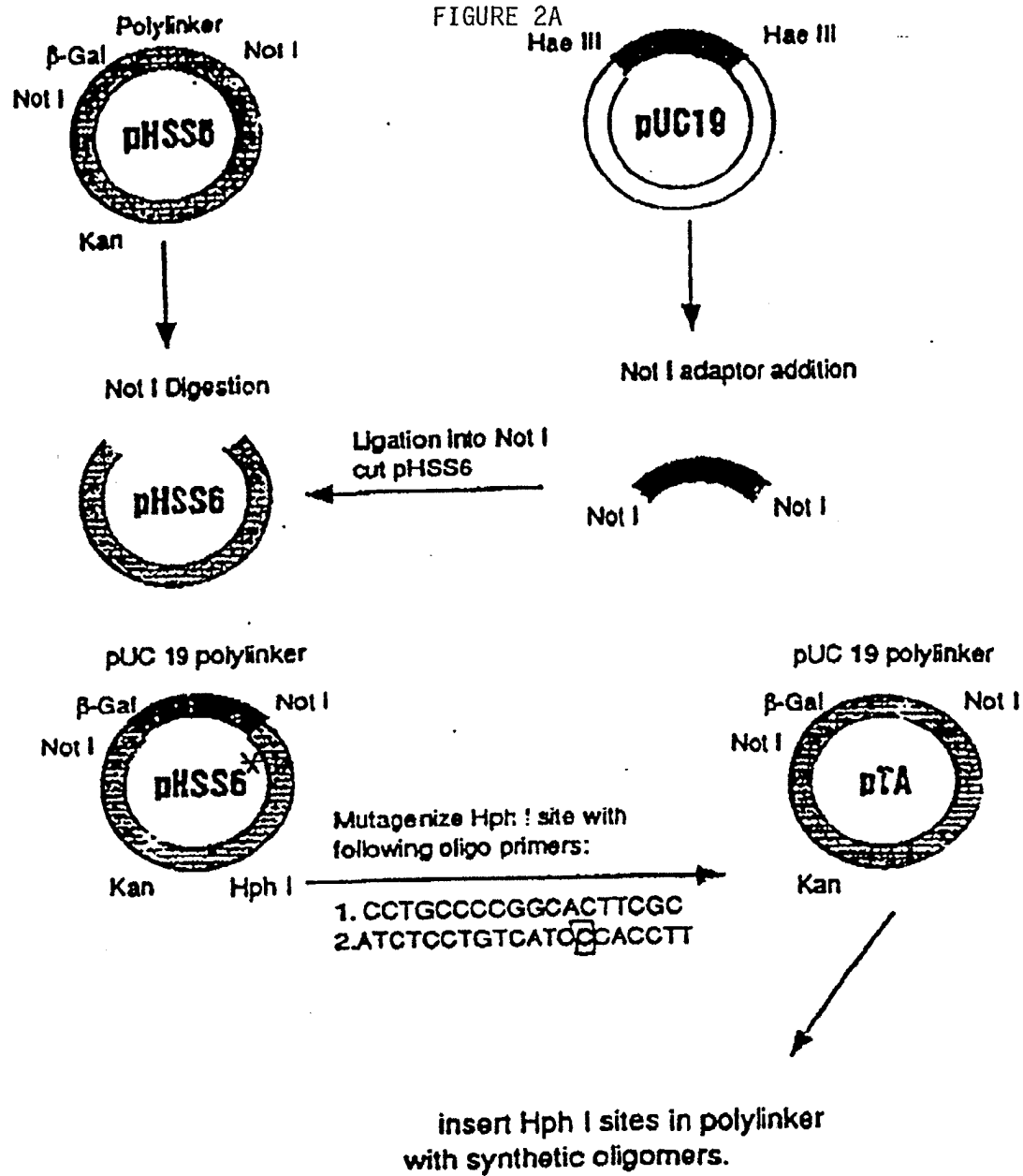
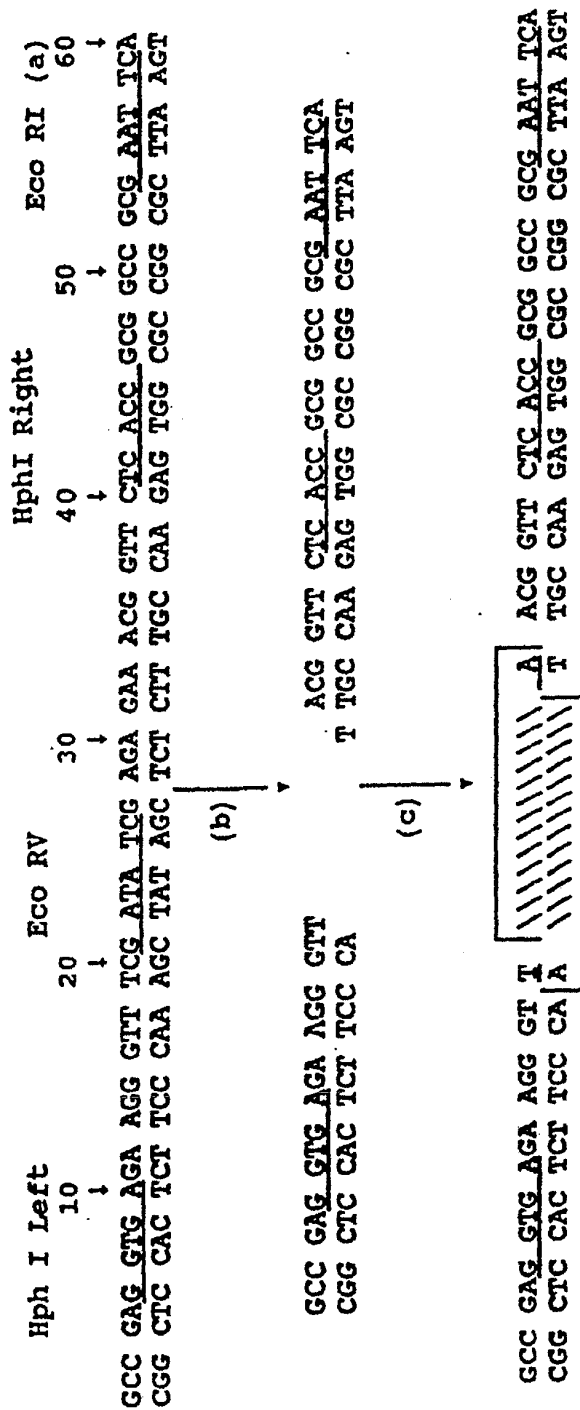


FIGURE 2B



(a) underlined regions indicate restriction recognition sequences

(b) Hph I digest, remove central fragment

(c) PCR amplified product (3'-dAMP) admixed; T4 DNA ligase.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07147

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5):C12N 15/00;C12P 19/34; C12Q 1/68; G01N 33/00, 33/48 U.S.CL.: 435/6,91,172.3,320.1,810; 436/63,94,501, 508											
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border: 1px solid black; text-align: left;">Classification System</th> <th style="border: 1px solid black; text-align: left;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">U.S.</td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">435/6,91,172.3, 320.1, 810; 436/63,94,501, 508</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	U.S.	435/6,91,172.3, 320.1, 810; 436/63,94,501, 508					
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U.S.	435/6,91,172.3, 320.1, 810; 436/63,94,501, 508										
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border: 1px solid black; text-align: left;">Category [*]</th> <th style="border: 1px solid black; text-align: left;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="border: 1px solid black; text-align: left;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="border: 1px solid black; vertical-align: top; text-align: center; padding: 5px;">Y</td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">Nucleic Acids Research, Volume 16, Number 20, issued 1988, Clark. "Novel Non-Templated Nucleotide Addition Reactions Catalyzed by Procaryotic and Eucaryotic DNA Polymerases". pages 9677-9686. see entire document.</td> <td style="border: 1px solid black; vertical-align: top; text-align: center; padding: 5px;">1-12. 16-28</td> </tr> <tr> <td style="border: 1px solid black; vertical-align: top; text-align: center; padding: 5px;">Y</td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">Wu, "Methods in Enzymology" Volume 68. Recombinant DNA". published 1979 by Academic Press (NY). see pages 3-24 and 41-50.</td> <td style="border: 1px solid black; vertical-align: top; text-align: center; padding: 5px;">1-28</td> </tr> </table>			Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	Y	Nucleic Acids Research, Volume 16, Number 20, issued 1988, Clark. "Novel Non-Templated Nucleotide Addition Reactions Catalyzed by Procaryotic and Eucaryotic DNA Polymerases". pages 9677-9686. see entire document.	1-12. 16-28	Y	Wu, "Methods in Enzymology" Volume 68. Recombinant DNA". published 1979 by Academic Press (NY). see pages 3-24 and 41-50.	1-28
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>											
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; vertical-align: top; padding: 5px;"> Date of the Actual Completion of the International Search 07 January 1992 </td> <td style="width: 50%; border: 1px solid black; vertical-align: top; padding: 5px;"> Date of Mailing of this International Search Report <div style="text-align: right; font-size: 1.2em; font-weight: bold;">22 JAN 1992</div> </td> </tr> <tr> <td style="border: 1px solid black; vertical-align: top; padding: 5px;"> International Searching Authority ISA/US </td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;"> Signature of Authorized Officer <div style="display: flex; align-items: center;"> <div style="margin-right: 20px;">Lori Yuan</div> <div style="text-align: right;"> ebw </div> </div> </td> </tr> </table>			Date of the Actual Completion of the International Search 07 January 1992	Date of Mailing of this International Search Report <div style="text-align: right; font-size: 1.2em; font-weight: bold;">22 JAN 1992</div>	International Searching Authority ISA/US	Signature of Authorized Officer <div style="display: flex; align-items: center;"> <div style="margin-right: 20px;">Lori Yuan</div> <div style="text-align: right;"> ebw </div> </div>					
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